Protocol for Restriction Enzyme Digest, Ligation, and Transformation:

1. Add 1ug of the plasmid to a new clean eppendorf tube and fill the tube with water until 42.5ul is reached. Keep enzymes on ice and add 1ul of each to both to the eppendorf tube. Add 0.5ul of BSA (if needed) and 5.0ul of the correct digestion buffer (a). flick the mixture to mix contents.
    a. Use Nebcutter to find correct buffer to use.
2. Incubate the digests at 37C for 60min in a water bath (a) or heat room (b).
    a. Ensure that the entire volume of solutions is under the water.
    b. Place it on a shaker or periodically mix the digest.
3. Incubate the three restriction digests at 80C in a water bath for 20min (a).
    a. Temperature need to heat inactivate enzymes is variable, in some cases 65C is enough. Check Nebcutter.
4. Refer to ligation calculator on the mixture you should use for the ligation (a,b) and then flick it to mix it.
    a. Keep T4 DNA ligase in a ice block to keep it around -20.
    b. The ligation buffer is located in -80C and put a dot on the container after using some of it.
5. Incubate the reaction mixture for 60-120min at room temperature, be sure to periodically flick the mixture to ensure it's mixed.
6. Heat inactivate the T4 DNA ligase by placing the mixture into a water bath at 80C for 20 min.
7. Obtain competent DH5alpha cells and thaw them on ice. Add ~2ul of the heat inactivated ligase mixture. Place the cells in 42C for 45secs and then return them back on ice for 2min. After, add 500-1000ul of lb and place the cells at 37C for 2 hours on a shaker.
8. Plate 150ul of cells onto the correct antibiotic.